

Short communication

Expression of the avian-specific toll-like receptor 15 in chicken heterophils is mediated by Gram-negative and Gram-positive bacteria, but not TLR agonists

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ABSTRACT

Toll-like receptors (TLRs) are a critical component of the innate immune response of mammalian and avian species. While most mammalian TLRs have been well characterized, the chicken-specific TLR15 has not been extensively studied. We recently demonstrated that TLR15 is differentially expressed between *Salmonella*-susceptible-and-resistant chickens, indicating a potential role in the innate immune response to infection with *Salmonella*. The aim of the present study was to gain better insight into the nature of the ligand for TLR15 by characterizing gene expression patterns of TLR15 by heterophils in response to numerous bacterial-derived TLR agonists LPS, flagellin, CpG oligodeoxynucleotides, lipotechoic acid (LTA), peptidoglycan (PGN), and Pam3CSK4 (PAM), stimulation with live *Salmonella enterica* serovar Enteritidis (SE-used as a positive control), chicken isolates of *Escherichia coli* (EC) and *Enterococcus gallinarum* (EG), the equine-specific pathogen *Rhodococcus equi*, and stimulation with heat-killed, and formalin-killed SE, EC, and EG. TLR15 expression increased significantly in response to stimulation with live, heat-killed and formalin-killed SE, EC, and EG, but was unaffected by stimulation with known TLR agonists and *R. equi*. Overall, these observations demonstrate that the individual TLR agonists are not the ligand for TLR15, and that TLR15 recognizes a unique, non-secreted, heat-stable component of both Gram-negative and Gram-positive bacteria commonly found in and/or capable of causing disease in chickens.

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1. Introduction

Pattern recognition receptors (PRRs) are a critical component of innate immune recognition in both mammals and chickens. Of the various types of PRR, the Toll-like receptor (TLR) family has been the most widely characterized. There are numerous TLRs in both mammals and chickens that recognize a wide array of bacterial and viral components. To date, 10 TLRs have been identified in chickens (TLR1 (types 1 and 2), TLR2 (types 1 and 2), TLR3, TLR4,

TLR5, TLR7, TLR15, and TLR21) (Boyd et al., 2001; Fukui et al., 2001; Higgs et al., 2006; Iqbal et al., 2005; Kaiser, 2007; Kestra et al., 2007; Philbin et al., 2005; Roach et al., 2005; Yilmaz et al., 2005). The function of TLRs is related to their ability to recognize conserved chemical structures called pathogen-associated molecular patterns (PAMPs). Numerous bacterial PAMPs have been defined, and their respective TLRs have been identified. The most extensively studied TLR is TLR4, which recognizes LPS, a critical component of Gram-negative bacteria. TLR2 is involved in recognition of a variety of microbial components including lipopeptides from Gram-positive bacteria and zymosan from fungi (Takeda and Akira, 2005). Unmethylated CpG motifs of bacterial DNA are recognized by TLR9 in mammals and by an unknown receptor in the chicken. Bacterial

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flagellin is recognized by TLR5, which is found in both mammals and chickens.

A previous study in the chicken revealed the presence of TLR15, a novel, avian-specific TLR that has no mammalian counterpart (Higgs et al., 2006). TLR15 appears to be specific to the chicken, as homologues of TLR15 have not been found in searches of other available avian sequences (Higgs et al., 2006). However, as chicken is the only completely sequenced avian genome, this does not preclude the existence of TLR15 in other avian species. While TLR15 shares some identity with chicken TLR2 (30.1%) and TLR14 of fugu (29.2%) and zebrafish (29.7%), these identities, combined with the pattern in the phylogenetic tree, are insufficient to assign a 1:1 orthology. Thus, chicken TLR15 is distinct from all known TLRs (Higgs et al., 2006; Roach et al., 2005). The ligand for this receptor has not been determined, but there is substantial evidence that it recognizes some component of *Salmonella*. Higgs and colleagues demonstrated that mRNA expression of TLR15 increased in the tissues of *Salmonella enterica* serovar Typhimurium (ST) infected birds (Higgs et al., 2006). A study conducted in our laboratory demonstrated that in birds infected with *Salmonella enterica* serovar Enteritidis (SE), mRNA expression of TLR15 was significantly greater in the ceca than in uninfected birds (MacKinnon et al., 2009). In addition, we recently demonstrated that basal levels of mRNA expression of TLR15 were greater in heterophils from *Salmonella*-resistant chickens than in heterophils from *Salmonella*-susceptible chickens (Nerren et al., 2009). Moreover, the response of heterophils from resistant chickens to stimulation with SE was significantly greater than the response of heterophils from susceptible chickens. Collectively, these studies strongly suggest that TLR15 recognizes some component of, and plays a role in the avian immune response to, multiple serovars of *Salmonella*. It is unknown, however, what component of *Salmonella* is recognized by TLR15, and whether expression of TLR15 follows a similar pattern in response to other genera of bacteria. The fact that TLR15 appears to be unique to the chicken and is molecularly distinct from all other known TLRs (Higgs et al., 2006; Roach et al., 2005) suggests a role in the defense against avian pathogens.

The specific objectives of this study were to: (1) determine whether bacterial-derived TLR agonists could stimulate mRNA expression of TLR15; (2) determine whether other (non-*Salmonella*) species of bacteria could stimulate mRNA expression of TLR15; and (3) determine whether mRNA expression patterns were similar among heterophils stimulated with live or dead bacteria.

2. Materials and methods

2.1. Experimental chickens

Viable eggs were obtained from a commercial breeder at day 18 of embryonation, transferred to hatchers (Petersime), and maintained under the same temperature and humidity conditions until hatch. At hatch, birds were housed in floor pens with pine shavings and were provided water and a balanced, unmedi-

cated ration ad libitum. Chicks were routinely tested for the absence of *Salmonella* and *Campylobacter*. The feed ration contained or exceeded the levels of critical nutrients recommended by the National Research Council (1994).

2.2. Cells, bacteria, and reagents

A poultry isolate of *Salmonella enterica* serovar Enteritidis (SE) (#97-11771) was obtained from the National Veterinary Services Laboratory (Ames, IA, USA). In house isolates of *Escherichia coli* (EC) and *Enterococcus gallinarum* (EG) were obtained directly from chickens. A virulent strain of *Rhodococcus equi* (RE) (ATCC 33701p+) was kindly provided by Dr. Noah Cohen (College of Veterinary Medicine, Texas A&M University). EC, SE, EG were cultured in tryptic soy broth (Difco Laboratories, Becton Dickinson Co., Sparks, MD, USA) overnight at 41 °C to stationary phase. Stock SE, EG, and EC (1×10^9 cfu/ml) were prepared as previously described (Swaggerty et al., 2003). RE was cultured in brain-heart infusion broth (Difco Laboratories) overnight at 37 °C with rotation (60 rpm), washed twice and re-suspended in sterile PBS (1×10^9 cfu/ml). Fresh stocks of bacteria were prepared for each set of experiments. Heat-killed SE, EG, and EC were prepared by heating the bacteria at 75 °C for 5 min. Formalin-killed SE, EG, and EC were prepared by incubating with 0.5% formalin in PBS overnight at 4 °C. The bacteria were then washed 3× with PBS and re-suspended to a concentration of 1×10^9 cfu/ml. The absence of live bacteria in the heat-killed and formalin-killed preparations was confirmed by streaking each of the preparations onto tryptic soy agar plates and incubating overnight at 41 °C.

2.3. Isolation and stimulation of heterophils

Blood from 50 two-day-old chickens was collected, pooled, and heterophils were purified as previously described (Kogut et al., 2003). Each blood collection and heterophil isolation was conducted on six separate days such that there were six biological replicates. Briefly, EDTA anti-coagulated blood was mixed with 1% methylcellulose (Sigma Chemical Co., St. Louis, MO, USA) and centrifuged at $25 \times g$ for 15 min to remove erythrocytes. The serum and buffy coat layers were retained and mixed with Ca^{2+} , Mg^{2+} -free Hanks' balanced salt solution (HBSS, 1:1; Sigma Chemical Co.). This mixture was layered over a discontinuous Ficoll-Hypaque (Sigma Chemical Co.) gradient (specific gravity 1.077 over specific gravity 1.119), and centrifuged at $250 \times g$ for 60 min. After centrifugation, the 1.119 band containing the heterophils was removed and washed once with HBSS. After washing, the cells were re-suspended in HBSS to a final concentration of 1×10^7 cells/ml and stored on ice until use. Cell preparations obtained by this method were typically >98% pure and >95% viable (Kogut et al., 2003).

All bacteria were used at a multiplicity of infection of 20:1 (20 bacteria:1 heterophil). Heterophils (1×10^7 cells/ml) were incubated for 1 h at 41 °C with their respective treatments. Following stimulation, cells were pelleted by centrifugation at $3000 \times g$ for 5 min. The super-

nant was discarded and cells were lysed by the addition of RLT buffer (Qiagen, Valencia, CA, USA). The lysates were stored at -20°C for future RNA extraction.

The following concentrations of TLR agonists were used in this study: lipopolysaccharide (LPS, $5\text{ }\mu\text{g/ml}$); flagellin (FGN, 200 ng/ml); lipotechoic acid (LTA), palmitoyl-3-cysteine-serine-lysine-4 (PAM), and peptidoglycan (PGN) (20 ng/ml); and CpG ($10\text{ }\mu\text{g/ml}$) (He et al., 2007; Kogut et al., 2005). Heterophils (1×10^7 cells/ml) were incubated with the TLR agonists as described above. For all treatments, non-stimulated heterophils were included as negative controls, and heterophils incubated with live SE were included as positive controls.

2.4. RNA isolation

RNA was extracted from lysed heterophils using an RNeasy Mini kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions. Genomic DNA contamination was removed using Turbo DNA-free (Ambion, Austin, TX, USA) following manufacturer's instructions. Total RNA was stored at -80°C until cDNA synthesis.

2.5. Real-time quantitative PCR

Chicken-specific primers and probes used in this study were designed as previously described (Nerren et al., 2009). Following DNase digestion, 500 ng total RNA was reverse transcribed in a $10\text{ }\mu\text{l}$ reaction using the ThermoScript RT-PCR system (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. cDNA was then diluted with DNase-free water to a working concentration of $10\text{ ng}/\mu\text{l}$. Each $20\text{-}\mu\text{l}$ real-time PCR reaction contained the following: $2.0\text{ }\mu\text{l}$ cDNA; 900 nM each forward (GAPDH-GGGCACGCCATCACTATCTTC; TLR15-CCATGCTTCCTGACCTACCTATCT) and reverse (GAPDH-ACCTGCATCTGCCATTGAT; TLR15-GTTAAGTTGGAGAGATTAAACCTAGGG) primer; 250 nM TaqMan MGB probe (GAPDH-(FAM) CAGGAGCGTGACCCC; TLR15-(FAM) CTCTGCAGCAACTTGAC) $10\text{ }\mu\text{l}$ $1\times$ TaqMan Universal Master Mix (Applied Biosystems); and molecular-grade water. Amplification and data analysis were carried out on a GeneAmp 7900 Sequence Detection System (Applied Biosystems). The thermal profile consisted of an initial hold at 50°C for 2 min, followed by a single denaturation at 95°C for 10 min, and then 40 cycles of 95°C for 15 s, 60°C for 60 s. Each PCR reaction was performed in duplicate.

The resulting threshold cycle (Ct—the PCR cycle at which amplification becomes significant enough to cross the predetermined threshold) values were normalized to the endogenous control, GAPDH, and the relative quantification values were determined using the ddCt method (Livak and Schmittgen, 2001). To assess differences between different treatments, ddCt values (i.e., the difference in Ct value between non-stimulated and SE-stimulated heterophils) were compared. Results were expressed as fold-change in TLR15 expression of stimulated heterophils relative to non-stimulated heterophils.

2.6. Statistical analysis

Statistical analysis on mean values from six independent experiments performed using a One-Way Repeated Measures Analysis of Variance using Sigma Stat software program (Jandel Scientific). A *P*-value <0.05 was considered statistically significant.

3. Results and discussion

In order to gain a better understanding of the role TLR15 plays in the immune response of the chicken, it is necessary to determine the ligand recognized by TLR15, and such knowledge will open the field to a multitude of studies designed to characterize this seemingly important receptor. However, because TLR15 is both novel and molecularly distinct from other known TLRs, it is difficult to determine a starting point for screening potential ligands. If TLR15 were expressed exclusively in certain tissues (for example only in the gastrointestinal tract), a logical starting point would be to screen bacteria/toxins involved in enteric disease. For example, TLR11 in mouse is predominantly expressed in the kidney and bladder, which led investigators to hypothesize that this receptor was involved in the immune response to pathogens of the urinary tract. Indeed, uropathogenic *E. coli* demonstrated strong activation of NF- κ B in TLR11 transfected HEK293 cells (Zhang et al., 2004). Unfortunately, TLR15 is expressed at various levels in multiple tissues throughout the body rather than being limited to a single site (Higgs et al., 2006). While our ultimate goal is to determine the ligand for TLR15, the primary goal of this study was to characterize mRNA expression patterns of TLR15 in response to known TLR ligands and different species of bacteria in order to gain a better understanding of the nature of the ligand for TLR15.

Our initial goal was to determine whether bacterial-derived TLR agonists stimulated mRNA expression of TLR15. Although mRNA expression is not a guaranteed predictor of ligand recognition by TLRs, there is substantial evidence demonstrating that mRNA expression of numerous TLRs increases in response to ligand recognition (Adamo et al., 2004; Ajuwon et al., 2009; Deng et al., 2009; Kobayashi et al., 2009). In addition to testing the TLR agonists that are known components of *Salmonella*, FGN (TLR5 agonist) and LPS (TLR4 agonist), we also tested several TLR2 agonists (i.e., PGN, LTA, and PAM) that are typically (although not exclusively) more prominent in Gram-positive bacteria (Murray et al., 2002). There has been some speculation that TLR15 may recognize the same (or a similar) ligand as TLR2, as gene expression patterns in TLR2 and TLR15 mRNA were highly similar in *Salmonella*-infected chicks (Higgs et al., 2006; MacKinnon et al., 2009). Heterophils stimulated with live SE respond by significantly increasing mRNA expression of TLR2 in magnitude similar to that of TLR15 (Nerren and Kogut, unpublished). Finally, we tested the mammalian TLR9 agonist, unmethylated CpG, because it is known to stimulate the immune response of chickens through an unknown receptor (He et al., 2007; Xie et al., 2003), and is a substantial component of the *Salmonella* genome (Dalpke et al., 2006).

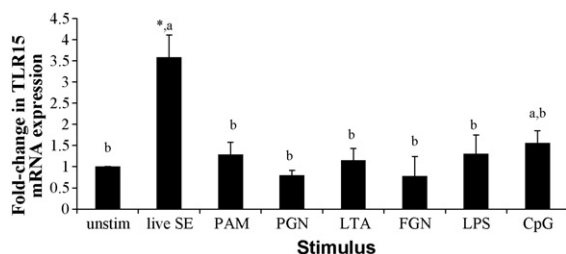


Fig. 1. Effect of bacterial-derived TLR agonists on mRNA expression of TLR15 by chicken heterophils. Unstimulated heterophils were used as the negative control, and live SE was used as the positive control. Bars with asterisks denote a significant ($P < 0.05$) difference from unstimulated control heterophils. Bars with different letters denote a significant ($P < 0.05$) difference between treatments (i.e., CpG treated heterophils were not significantly different from unstimulated heterophils, SE-stimulated heterophils, or any other of the TLR agonist stimulated heterophils).

Heterophils stimulated with live SE (positive control) had significantly ($P < 0.05$) greater expression of TLR15 mRNA than heterophils stimulated with the TLR agonists, with the exception of CpG stimulated heterophils (Fig. 1). When mRNA expression among heterophils stimulated with the various TLR agonists was compared, however, no significant differences in mRNA expression of TLR15 were observed (Fig. 1). When compared to unstimulated heterophils, there were no significant differences in mRNA expression of TLR15 by heterophils stimulated with the various agonists (Fig. 1). In contrast, there was a significant ($P < 0.05$) increase in expression by heterophils stimulated with live SE (positive control) when compared to unstimulated heterophils (Fig. 1). While it is possible that TLR15 recognizes some combination of these TLR agonists, these data suggest that the individual agonists themselves are not the ligand for this receptor.

Because TLR15 appears to exist only in the chicken, it is plausible that it has evolved to contribute to the immune defense against specific avian pathogens. We stimulated heterophils with three other types of bacteria and examined changes in mRNA expression of TLR15. Our objectives were to determine the following: (1) whether TLR15 recognized other related Gram-negative bacteria or was exclusive to *Salmonella*; (2) whether TLR15 recognized a Gram-positive organism; and (3) whether TLR15 recognized an organism not known to be isolated from or cause disease in chickens. We chose to stimulate heterophils with *E. coli* (EC) because, like *Salmonella*, it is a member of the Enterobacteriaceae family, is a commensal organism of chickens, and is the etiologic agent of avian colibacillosis (Dziva and Stevens, 2008). *E. gallinarum* (EG) was chosen as a stimulus because it is a Gram-positive organism frequently isolated in chickens (Swaggerty et al., 2005). *R. equi* (RE) was selected because it is primarily associated with disease in horses and humans (Meijer and Prescott, 2004), and has never been reported to cause infection in the chicken. Although one study reported that RE was isolated from 4% of fecal samples from poultry farms, these isolates were obtained from droppings and were likely environmental contaminants, as RE is a ubiquitous soil-saprophyte (Carman and Hodges, 1987; Meijer and Prescott, 2004).

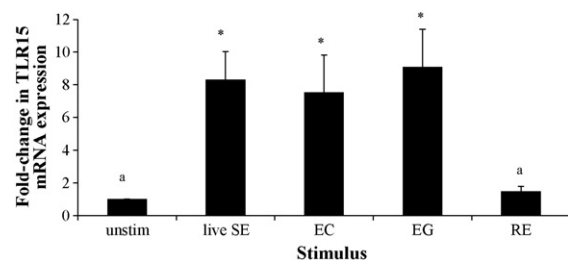


Fig. 2. Effect of different species of bacteria on mRNA expression of TLR15 by chicken heterophils (SE: *S. enteritidis*; EC: *E. coli*; EG: *E. gallinarum*; RE: *R. equi*). Asterisks denote a significant ($P < 0.05$) difference from unstimulated control heterophils. Letters denote a significant ($P < 0.05$) a significant difference between treatments (i.e., RE was significantly different from EG, but not significantly different from unstimulated heterophils).

We compared mRNA expression of TLR15 by unstimulated heterophils to heterophils stimulated with live SE (positive control), live EC, live EG, and live RE. When compared to unstimulated heterophils, heterophils stimulated with bacteria commonly isolated from chickens (SE, EC, and EG) all had significantly ($P < 0.05$) greater expression of TLR15 mRNA (Fig. 2). In contrast, mRNA expression of TLR15 by heterophils stimulated with RE was not significantly increased when compared to unstimulated heterophils (Fig. 2). There were no significant differences in TLR15 expression between heterophils stimulated with live SE (positive control), EC, and EG. Levels of TLR15 mRNA expression by heterophils stimulated with RE, however, were significantly lower ($P < 0.05$) than levels of TLR15 mRNA expression by heterophils stimulated with live SE (positive control), EC, and EG (Fig. 2). While the finding that RE was the only bacteria that did not induce mRNA expression of TLR15 is not conclusive evidence that TLR15 recognizes only bacteria commonly isolated from (or capable of causing disease in) the chicken, it supports the hypothesis that its role is in the defense against avian pathogens. This hypothesis could be evaluated by testing with other bacteria known to be specific to non-avian hosts.

Our next objective was to determine whether viable and/or intact bacteria were required to induce TLR15 expression. Thus, we compared mRNA expression levels of unstimulated heterophils to heterophils stimulated with heat-killed and formalin-killed strains of SE, EC, and EG. When compared to unstimulated heterophils, we found significantly increased levels ($P < 0.05$) of TLR15 mRNA expression by heterophils stimulated with both heat-killed and formalin-killed of SE, EC, and EG (Fig. 3). There was no significant difference between preparations of bacteria in levels of TLR15 mRNA expression by heterophils. The finding that killed bacteria were able to induce TLR15 mRNA expression of the same magnitude as live bacteria suggests that whatever component of SE is stimulating this expression it is not actively secreted by the bacterium. This finding is important because *Salmonellae*, along with many other Gram-negative pathogens, utilize numerous secreted proteins designed to interfere with host defense, thereby allowing invasion, survival, and replication inside the host (Samudrala et al., 2009). Of particular importance are the type III secretion system proteins (T3SS),

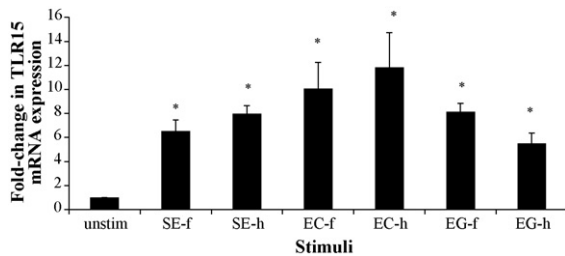


Fig. 3. Effect of formalin-killed and heat-killed *Salmonella enteritidis* (SE-f and SE-h, respectively), *Escherichia coli* (EC-f and EC-h), and *Enterococcus gallinarum* (EG-f and EG-h) on mRNA expression of TLR15 by chicken heterophils. Asterisks denote a significant ($P < 0.05$) difference from unstimulated heterophils.

which are major virulence factors that contribute to the colonization of *Salmonella* in both mammals and chickens (Dieye et al., 2009). As heat-treatment denatures heat-labile bacterial proteins, rendering them inactive and in many cases unrecognizable by their cognate receptors, the ability of heat-killed bacteria to induce TLR15 expression suggests that the molecule recognized by TLR15 is heat-stable antigen. In addition, heat-treatment also has been demonstrated to substantially reduce phagocytosis of numerous species of bacteria (including *Salmonella*) by neutrophils and macrophages, which suggests that ligand recognition of TLR15 mRNA is not dependent on phagocytosis (DeChatelet et al., 1974; Tomita et al., 1981).

In summary, we have demonstrated that mRNA expression of TLR15 increases in response to stimulation with multiple genera of bacteria commonly isolated from chickens, as well as heat-killed and formalin-killed preparations of these bacteria, but is unresponsive to stimulation with known TLR agonists of bacterial origin and to *R. equi*, a bacterium that is not found in chickens. These findings demonstrate that the ligand for TLR15 is a unique, non-secreted, heat-stable component of multiple genera of both Gram-positive and Gram-negative bacteria. The finding that TLR15 mRNA is not differentially expressed in response to *R. equi* provides evidence that this avian-specific receptor may have evolved as a broad spectrum TLR involved in the immune response to bacteria that have the potential to cause disease in chickens. Although increased mRNA expression levels do not necessarily correlate with ligand recognition, the fact that numerous TLRs respond to their respective ligands by increasing mRNA expression suggests that the observations made in this study are indicative of the nature of the ligand for TLR15, and provide valuable insight for future experiments aimed at determining this molecule.

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purpose of providing specific information and is not a recommendation or endorsement by the USDA.

References

- Adamo, R., Sokol, S., Soong, G., Gomez, M.I., Prince, A., 2004. *Pseudomonas aeruginosa* flagella activate airway epithelial cells through asialoGM1 and toll-like receptor 2 as well as toll-like receptor 5. *Am. J. Respir. Cell Mol. Biol.* 30, 627–634.
- Ajuwon, K.M., Banz, W., Winters, T.A., 2009. Stimulation with peptidoglycan induces interleukin 6 and TLR2 expression and a concomitant downregulation of expression of adiponectin receptors 1 and 2 in 3T3-L1 adipocytes. *J. Inflamm.* 6, 8.
- Boyd, Y., Goodchild, M., Morroll, S., Bumstead, N., 2001. Mapping of the chicken and mouse genes for toll-like receptor 2 (TLR2) to an evolutionarily conserved chromosomal segment. *Immunogenetics* 52, 294–298.
- Carman, M.G., Hodges, R.T., 1987. Distribution of *Rhodococcus equi* in animals, birds, and from the environment. *N. Z. Vet. J.* 35, 114–115.
- Dalpe, A., Frank, J., Peter, M., Heeg, K., 2006. Activation of toll-like receptor 9 by DNA from different bacterial species. *Infect. Immun.* 24, 940–946.
- DeChatelet, L.R., Mullikin, D., Shirley, P.S., McCall, C.E., 1974. Phagocytosis of live versus heat-killed bacteria by human polymorphonuclear leukocytes. *Infect. Immun.* 10, 25–29.
- Deng, F., Han, F., Wu, C., 2009. Expression and immune effect of toll-like receptor 4 in human trophoblast cells. *J. Huazhong Univ. Sci. Technol. Med. Sci.* 29, 359–362.
- Dieye, Y., Ameiss, K., Mellata, M., Curtiss, R., 2009. The *Salmonella* pathogenicity island (SPI) 1 contributes more than SPI2 to the colonization of the chicken by *Salmonella enterica* serovar Typhimurium. *BMC Microbiol.* 9, 3.
- Dziva, F., Stevens, M.P., 2008. Colibacillosis in poultry: unraveling the molecular basis of virulence of avian pathogenic *Escherichia coli* in their natural hosts. *Avian Pathol.* 37, 355–366.
- Fukui, A., Inoue, N., Matsumoto, M., Nomura, M., Yamada, K., Matsuda, Y., Toyoshima, K., Seya, T., 2001. Molecular cloning and functional characterization of chicken toll-like receptors. A single chicken toll covers multiple molecular patterns. *J. Biol. Chem.* 276, 47143–47149.
- He, H., Genovese, K.J., Swaggerty, C.L., Nisbet, D.J., Kogut, M.H., 2007. *In vivo* priming heterophils innate immune functions and increasing resistance to *Salmonella enteritidis* infection in neonatal chickens by immune stimulatory CpG oligodeoxynucleotides. *Vet. Immunol. Immunopathol.* 117, 275–283.
- Higgs, R., Cormican, P., Cahalane, S., Allan, B., Lloyd, A.T., Meade, K., James, T., Lynn, D.J., Babiuk, L.A., O'Farrelly, C., 2006. Induction of a novel chicken toll-like receptor following *Salmonella enterica* serovar typhimurium infection. *Infect. Immun.* 74, 1692–1698.
- Iqbal, M., Philbin, V.J., Withanage, G.S., Wigley, P., Beal, R.K., Goodchild, M.J., Barrow, P., McConnell, I., Maskell, D.J., Young, J., Bumstead, N., Boyd, Y., Smith, A.L., 2005. Identification and functional characterization of chicken toll-like receptor 5 reveals a fundamental role in the biology of infection with *Salmonella enterica* serovar Typhimurium. *Infect. Immun.* 73, 2344–2350.
- Kaiser, P., 2007. The avian immune genome—a glass half-full or half-empty? *Immunology* 117, 221–230.
- Keestra, A.M., de Zoete, M.R., van Aubel, R.A., van Putten, J.P., 2007. The central leucine-rich repeat region of chicken TLR16 dictates unique ligand specificity and species-specific interaction with TLR2. *J. Immunol.* 178, 7110–7119.
- Kogut, M.H., Rothwell, L., Kaiser, P., 2003. Differential regulation of cytokine gene expression by avian heterophils during receptor-mediated phagocytosis of opsonized and nonopsonized *Salmonella enteritidis*. *J. Interferon Cytokine Res.* 23, 319–327.
- Kogut, M.H., Iqbal, M., He, H., Philbin, V., Kaiser, P., Smith, A., 2005. Expression and function of Toll-like receptors in chicken heterophils. *Dev. Comp. Immunol.* 29, 791–807.
- Kobayashi, M., Yoshiki, R., Sakabe, J., Kabashima, K., Nakamura, M., Tokura, Y., 2009. Expression of toll-like receptor 2, NOD2 and dectin-1 and stimulatory effects of their ligands and histamine in normal human keratinocytes. *Br. J. Dermatol.* 160, 297–304.
- Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-(delta delta C(T))} method. *Methods* 25, 402–408.
- MacKinnon, K.M., He, H., Nerren, J.R., Swaggerty, C.L., Genovese, K.J., Kogut, M.H., 2009. Expression profile of toll-like receptors within the gastrointestinal tract of 2-day-old *Salmonella enteritidis*-infected broiler chickens. *Vet. Microbiol.* 137, 313–319.
- Meijer, W.G., Prescott, J.F., 2004. *Rhodococcus equi*. *Vet. Res.* 35, 383–396.

- Murray, P.R., Rosenthal, R.S., Kobayashi, G.S., Pfaller, M.A. (Eds.), 2002. Medical Microbiology, 4th edition. Mosby, Inc., St. Louis, MO, pp. 266–280.
- National Research Council, 1994. Nutritional Requirements of Poultry, 9th edition. National Academy Press, Washington, DC.
- Nerren, J.R., Swaggerty, C.L., MacKinnon, K.M., Genovese, K.J., He, H., Pevzner, I., Kogut, M.H., 2009. Differential mRNA expression of the avian-specific toll-like receptor 15 between heterophils from *Salmonella*-susceptible and -resistant chickens. *Immunogenetics* 61, 71–77.
- Philbin, V.J., Iqbal, M., Boyd, Y., Goodchild, M.J., Beal, R.K., Bumstead, N., Young, J., Smith, A.L., 2005. Identification and characterization of a functional, alternatively spliced toll-like receptor 7 (TLR7) and genomic disruption of TLR8 in chickens. *Immunology* 114, 507–521.
- Roach, J.C., Glusman, G., Rowen, L., Kaur, A., Purcell, M.K., Smith, K.D., Hood, L.E., Aderem, A., 2005. The evolution of vertebrate toll-like receptors. *Proc. Natl. Acad. Sci. U.S.A.* 102, 9577–9582.
- Samudrala, R., Heffron, F., McDermott, J.E., 2009. Accurate prediction of secreted substrates and identification of a conserved putative secretion signal for type III secretion systems. *PLoS Pathogens*, 5.
- Swaggerty, C.L., Pevzner, I.Y., Ferro, P.J., Crippen, T.L., Kogut, M.H., 2003. Association between *in vitro* heterophil function and the feathering gene in commercial broiler chickens. *Avian Pathol.* 32, 483–488.
- Swaggerty, C.L., Lowry, V.K., Ferro, P.J., Pevzner, I.Y., Kogut, M.H., 2005. Disparity in susceptibility to vancomycin-resistant *Enterococcus* organ invasion in commercial broiler chickens that differ in innate immune responsiveness. *Food Agric. Immunol.* 16, 1–15.
- Takeda, K., Akira, S., 2005. Toll-like receptors in innate immunity. *Int. Immunol.* 17, 1–14.
- Tomita, T., Blumenstock, E., Kanegasaki, S., 1981. Phagocytic and chemiluminescent responses of mouse peritoneal macrophages to living and killed *Salmonella typhimurium* and other bacteria. *Infect. Immun.* 32, 1242–1248.
- Xie, H., Raybourne, R.B., Babu, U.S., Lillehoj, H.S., Heckert, R.A., 2003. CpG-induced immunomodulation and intracellular bacterial killing in a chicken macrophage cell line. *Dev. Comp. Immunol.* 27, 823–834.
- Yilmaz, A., Shen, S., Adelson, D.L., Xavier, S., Zhu, J.J., 2005. Identification and sequence analysis of chicken toll-like receptors. *Immunogenetics* 56, 743–753.
- Zhang, D., Zhang, G., Hayden, M.S., Greenblatt, M.B., Bussey, C., Flavell, R.A., Ghosh, S., 2004. A toll-like receptor that prevents infection by uropathogenic bacteria. *Science* 303, 1522–1526.